Social interactions modulate the virulence of avian malaria infection

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Abstract

There is an increasing understanding of the context-dependent nature of parasite virulence. Variation in parasite virulence can occur when infected individuals compete with conspecifics that vary in infection status; virulence may be higher when competing with uninfected competitors. In vertebrates with social hierarchies, we propose that these competition-mediated costs of infection may also vary with social status. Dominant individuals have greater competitive ability than competing subordinates, and consequently may pay a lower prevalence-mediated cost of infection. In this study we investigated whether costs of malarial infection were affected by the occurrence of the parasite in competitors and social status in domestic canaries (*Serinus canaria*). We predicted that infected subordinates competing with non-infected dominants would pay higher costs than infected subordinates competing with infected dominants. We also predicted that these occurrence-mediated costs of infection would be ameliorated in infected dominant birds. We found that social status and the occurrence of parasites in competitors significantly interacted to change haematocrit in infected birds. Namely, subordinate and dominant infected birds differed in haematocrit depending on the infection status of their competitors. However, in contrast to our prediction dominants fared better with infected subordinates, whereas subordinates fared better with uninfected dominants. Moreover, we found additional effects of parasite occurrence on mortality in canaries. Ultimately, we provide evidence for costs of parasitism mediated by social rank and the occurrence of parasites in competitors in a vertebrate species. This has important implications for our understanding of the evolutionary processes that shape parasite virulence and group living.

Keywords:
Avian malaria, competition, group living, social rank, virulence, social stress, Plasmodium relictum, SGS1

1. **Introduction**

The ubiquity of parasites ensures that the ability to minimize costs of infection is one of the major factors affecting an organism's fitness. Hosts vary in the degree of damage suffered when exposed to a similar parasitic challenge, and assessing the factors which determine these differences in parasite-mediated morbidity and mortality (generally called parasite virulence) is of fundamental interest to evolutionary biologists (Alizon et al. 2009). Parasite virulence is affected both by host genotype, parasite genotype and their interaction (Grech et al. 2006; Lefevre et al. 2007). As well as genetic differences, environmental conditions can alter parasite virulence (e.g. Jokela et al. 1999; Ferguson and Read 2002; Bedhomme et al. 2004; Tseng 2006), and individual differences in physiological conditions (e.g. levels of host physiological stress) can alter the magnitude of the cost of infection (Brown et al. 2000).

One factor influencing parasite virulence, which has been experimentally demonstrated, is additive costs of parasitism through modification of host competitive ability (Hochberg 1998). Here, the effects of a parasitic infection are not only determined by parasitism of the focal host, but also by parasitism of the host's conspecific competitors. Bedhomme et al. (2005) showed that when larvae of the mosquito *Aedes aegypti* were infected with the microsporidian parasite *Vavraia culicis* they had a longer developmental time, a demonstrable fitness cost in this species. However, this cost of parasitism was also dependent on the infection status of conspecifics: the developmental time was always longer for infected larvae competing with non-infected larvae, than for infected larvae competing with other infected individuals. This suggests that although competition between individuals is normally costly, the strength of this cost is determined by both individual parasitic intensity, and the prevalence of parasitism in conspecific competitors. This idea has been

confirmed in one plant species (Pagan et al. 2009) and two animal species (Bedhomme et al. 2005; Koprivnikar et al. 2008); however, the hypothesis is also likely to apply to many group living organisms.

Unlike plants or mosquito larvae, many vertebrates including birds live in social groups, and have a large behavioural repertoire. As such, interactions among individuals are likely to be very complex. In many group living birds, social hierarchies are established between dominant and subordinate individuals. In these cases, competition between individuals is often mediated by social rank. For example, in canaries it was previously shown that dominant birds have a greater access to food than subordinates, and subordinate birds avoid interactions with dominants at food sites (Parisot et al. 2004). For such social animals, not only may parasite virulence depend on the infection status of competitors, but also on their level of competitive ability (determined by social rank). We expect that the outcome of intraspecific competition may be influenced more by conspecific infection status for subordinate than dominant birds. Thus, we predict the competition-mediated increase in morbidity and mortality with infection to be more severe for subordinate than for dominant birds.

The goal of this study was to assess the interactive effects between infection, social status, and the occurrence of a parasite in social competitors on morbidity and mortality, using domestic canaries as hosts and *Plasmodium relictum* (lineage SGS1) an avian malarial parasite. By keeping canaries in flocks of 6 birds, and scoring for consistent dominant and subordinate behaviours, we classified birds as dominant (D) and subordinate (S). We had four treatment groups in flocks with birds either infected (+), or non-infected (-), with the *Plasmodium* parasite. These groups were: D+ S+; D+ S-; D- S+; and D- S-. Following infection, we measured mortality, and morbidity in terms of changes in body mass and

haematocrit. We also measured parasitaemia of blood samples post infection, based on a qPCR technique (Cellier-Holzem et al. 2010). We predicted significant three-way interactions among social status, occurrence of parasite in competitors, and infection status on mortality rate, and physiological changes thought to reflect parasite virulence. Our major specific prediction was that infected subordinate birds competing with uninfected dominant birds would suffer greater morbidity than infected subordinate birds competing with infected dominant birds. However, for infected dominant birds this difference in morbidity mediated by the infection status of competing subordinates would be ameliorated (or reduced) by their greater competitive ability.

2. **Materials and methods**

We used 96 adult male canaries during the experiment, sourced for us by a local provider and breeder. All of the canaries were adults and prior to commencement each bird was molecularly sexed following a standard PCR technique (Fridolffson and Ellegren 1999). We only used male canaries in the experiment as we did not wish to confound the experiment with differences between sexes, or by interactions in- and between pairs of birds. After confirming the sex of each bird, we divided them between16 aviaries (2.5 * 1.5 * 2.2 m), 6 birds per aviary. Each bird was weighed, and had its tarsus length measured prior to re-housing in a new flock.

2.1. Husbandry and competition

Before commencing the manipulation of competition, all cages were provided with ad libitum food (a commercial seed mix, lettuce and apple) for 7 days. Since we were interested in competition between birds, and previous studies have shown that limited food provision results in an increase in competition (Bedhomme et al. 2005; Hawley et al. 2006), following the 7 days of acclimation, the birds were provided each day with 12g of seeds per bird per day, provided in one circular feeding dish per cage. We had previously found that 12g of seeds is the maximum amount a single bird would eat per day (Larcombe et al. unpublished data). This amount of seeds was thus sufficient to nourish each bird, though encourage competition between birds (pers. obs.). During the course of the experiment, the cages were monitored daily, and if a bird died the amount of seed was reduced accordingly.

2.2. Behavioural observation

We performed behavioural observations to assess the social status of each bird before the experimental infection (though after being housed in the experimental flocks), and to

monitor any changes in social status related to the treatments. The first phase of observations was carried out 4 days after the start of the limited seed provision and 11 days after being placed in their flocks, by which time birds had established dominant and subordinate roles. We performed behavioural observations for 3 consecutive days. Each morning at 09.00 we removed the remaining seed from the previous day, and left cages for 30 minutes without seeds. Following the 30 minute food deprivation, we placed a seed feeder that allowed only a single bird to feed at a time in each cage. We also placed a video camera in each cage and filmed the interactions among birds at the feeder for 20 minutes, starting when the feeder was first entered. Birds were marked with non-toxic coloured pen on the back of the head or wings for identification on the video tapes.

To score the bird's behaviour, when the video was re-watched the 20 minute time period was divided into 10 two minute blocks. Birds were scored for the presence or absence of certain behaviours in each block. We counted the frequency of the following behaviours in the experimental trials: Primary Access (PA) to the feeder, where a bird successfully fed directly from the hole in the feeder: Secondary Access (SA), when a bird was motivated to feed, and appeared at the feeder, either attempting to feed, or pecking at discarded seeds, but did not achieve Primary Access; Aggression (AGG), where a bird aggressively postured towards another, typically by lowering its head and fanning and trembling its wings, or by pecking out at the other bird, sometimes escalating into a physical fight. All of these behavioural measures represent dominance (primary access and aggression) or subordination (secondary feeding).

It is clear that social hierarchies, even within those assumed to be linear, are often very complex. Here, we wished to compare "dominant" and "subordinate" birds in their reactions to infection. As such we required birds to be labelled prior to infection. We classified birds

within a flock into two categories, based on social status: 3 dominant birds, and 3 subordinate birds. Although this assumes that the third ranked bird in a cage is a 3rd dominant, as opposed to a 4th subordinate we believe this was justified. Classification was based on the mean number of primary accesses to the seeds across the first three days of behavioural observations. We based our social status classification on primary access as we felt this best reflected "dominance" *per se*, that is the ability to monopolise the food resource. After infection, to check our classifications were sound we created a ratio of primary to secondary access for the same three days as (PA day 1 + PA day 2 + PA day 3 +1) / (SA day 1 + SA day 2 + SA day 3 +1). In this case a ratio of ≥1 suggests a bird was dominant (spent more time primary feeding, than secondary feeding), with the opposite true for a ratio of < 1. The mean number of ≥1 birds per cage was 2.5± 0.29. Thus we believe our initial categorisation of 3 dominant vs 3 subordinate birds was sound. It is also important to note that daily primary access was highly positively correlated with daily aggression (spearman's $\rho > 0.716$, $p < 0.0001$ in all cases). Additionally, our behavioural scores were repeatable across the consecutive days measured (PA: spearman's $\rho > 0.582$, $p < 0.0001$ in all cases. AGG: spearman's $\rho > 0.457$ $p < 0.0001$ in all cases). We believe we have accurately described each bird as having a distinct, repeatable behavioural pattern. Measures such as frequency of aggression or submission have previously been used in avian behavioural studies (e.g. Torda et al. 2004; Müller et al., 2012), and it is important in classifying animals as having stable behavioural types that these must be repeatable across time (Sih and Bell, 2008). Cronbach's alpha, an internal consistency statistic that has previously been used to assess the stability of animal behaviour types (e.g. Budaev 1997; Budaev et al. 1999), was high for our measures (PA = 0.823, AGG =0.790) suggesting that each bird had a consistent behaviour pattern. To check that dominance was continuous throughout the experiment, we repeated observations

for three days following day 9. We did not find evidence that birds altered dominance throughout the experiment.

2.3. Experimental infection

We used the avian malaria parasite *Plasmodium relictum* (lineage SGS1) originally obtained from a natural population of house sparrows, and cross-transferred to naive canaries. Infected blood was cryopreserved and stored at -80°C (see details in Bichet et al. 2012). For the purpose of the present experiment, cryopreserved blood was thawed (Bichet et al. 2012) and transferred intraperitoneally to 5 domestic canaries. Eleven days post-infection (dpi), parasitaemia was evaluated from thin blood smears (absolute methanol fixation, 10% Giemsa staining, observation of 10,000 erythrocytes). Blood was collected from donors to prepare a stock suspension diluted in PBS containing the desired number of parasites per inoculum (1 x 10^6 asexual parasites) that served to infect birds.

On the day of infection, we captured all birds within a flock. Each bird was weighed, and a small volume of blood was taken in a capillary tube for subsequent haematocrit assessment. Finally, the bird was either injected with *Plasmodium*-infected canary blood, or with control uninfected canary blood, according to their dominance status as outlined below. We had four treatment schemes which were divided randomly within the aviary: Dominant infected with Subordinate infected (D+ S+); Dominant infected with Subordinate non-infected (D+ S-); Dominant non-infected with Subordinate infected (D- S+); and Dominant non-infected with Subordinate non-infected (D- S-). Therefore each individual of the same social rank competed against competitors that were either infected or not. Throughout the paper we will refer to the infection status of conspecific competitors as parasite occurrence. All birds of the same dominant status within a flock were treated identically i.e. in D+ flocks, every dominant bird was infected.

212 2.4. Post-infection monitoring

213 Following the experimental infection (day 0), birds were left in their flocks, and were
214 monitored at regular intervals. We re-caught all birds on days 5, 8, 11, 14 and 17 post-
215 infection. On each of these sampling days, we took a small blood sample for haematocrit
216 measurement and parasitaemia (qPCR), and we weighed each bird. The measurement of
217 haematocrit offered a good indication of the specific cost of infection, since a negative change
218 in haematocrit (the proportion of red cells in a given sample of blood) can be representative of
219 damage caused by malarial parasites in canaries (Spencer et al. 2005; Cellier-Holzem et al.
220 2011).

221 2.5. Mortality and ethical note

222 Some morbidity and mortality is an inherent part of studies involving experimental infections
223 of animals. This experiment was carried under the permit # 21-CAE-085 (departmental
224 veterinary services).

225 2.6. Assessing parasitaemia

226 Parasitaemia was assessed using a recently developed quantitative PCR assay (Cellier-
227 Holzem et . 2011, Bichet et al. 2012). For each individual we conducted two qPCR reactions
228 in the same run: one targeting the nuclear 18s rDNA gene of *Plasmodium* (Primers 18sPlasm7
229 (5'-AGC CTG AGA AAT AGC TAC CAC ATC TA-3'), 18sPlasm8 (5'-TGT TAT TTC
230 TTG TCA CTA CCT CTC TTC TTT-3'), and fluorescent probe Plasm Hyb2 (5'-6FAM-CAG
231 CAG GCG CGT AAA TTA CCC AAT TC-BHQ1-3'))and the other targeting the 18s rDNA
232 gene of bird (Primers 18sAv7 (5'-GAA ACT CGC AAT GGC TCA TTA AAT C-3'),
233 18sAv8 (5'-TAT TAG CTC TAG AAT TAC CAC AGT TAT CCA-3') and fluorescent probe
234 18sAv Hyb (5'-VIC-TAT GGT TCC TTT GGT CGC TC-BHQ1-3')).

Parasitaemia was calculated as relative quantification values (RQ) as $2^{-(Ct\ 18s\ \textit{Plasmodium} - Ct\ 18s\ Bird)}$ using the software SDS 2.2 (Applied Biosystem). Ct represents the number of PCR cycles at which fluorescence is first detected as statistically significant above the baseline and RQ can be interpreted as the fold-amount of target gene (*Plasmodium* 18s rDNA) with respect to the amount of the reference gene (host 18s rDNA). All qPCR reactions were carried out in an ABI Prism 7900 cycler (Applied Biosystem). RQ values were log-transformed prior to statistical analyses.

2.7. Statistic analysis

For body mass, haematocrit, and parasitaemia we constructed an identical GLMM using SAS (9.1.3). Data for parasitaemia (RQ values) were log transformed prior to analysis and thereafter all variables were modelled with a normal distribution. The models were fully factorial and included the fixed factors dominance status (dominant/subordinate), infection status (uninfected/infected) and parasite occurrence (competitors infected/competitors non-infected). Time was added to each model as a continuous fixed effect to examine mean changes over time, and time^2 was added to account for quadratic changes in each variable over time. We also included all possible interactions between these terms. Additionally we had three random factors. Bird identity nested within cage (bird(cage)) was added, as this allows the model to control for non-independence of birds housed in the same cage over the course of the experiment, and permitted the variance between birds to be estimated. We added cage as a random factor to estimate the variance between cages. We also used time as a random factor with bird(cage) as a subject, using an autoregressive type 1 covariance matrix to estimate within-individual variation, controlling for correlations between observations taken closer together in time. Baseline measures prior to the experiment were included for models of haematocrit and body mass. Since we found that mortality was generally higher in

dominant birds (see results) and this might impact our results for body mass and haematocrit, mortality and mortality*dominance were added to these models. For our models explaining parasitaemia we did not have a baseline, since parasitaemia is always zero pre-infection. We included haematocrit as a covariate in analyses of parasitaemia, since the proportion of parasite to host genes will depend on the number of host red blood cells in addition to the number of parasites in each sample. We also analyzed mortality using a simpler model. We tested the probability of mortality using a binary distribution, with infection, dominance, occurrence, and their interactions as fixed factors, and including cage as a random factor to control for the non-independence of birds grouped together. We also tested for differences in behaviour following our experimental treatments; however, behavioural tests were only conducted in one block post-infection. Therefore we analyzed the change in aggression for each bird (mean frequency of aggression pre-experiment – mean frequency post-experiment), using a GLMM with cage identity as a random factor. Non-significant terms were dropped from the models starting with higher-order interactions, until only significant terms remained. Throughout the results relevant statistics are reported from the final model, though statistics for non-significant terms of interest are reported from the point they were dropped from models. Degrees of freedom were corrected using the satterthwaite method. Three birds were excluded from our results as they died early in the experimental phase, as a result of haemorrhage non-attributable to our experimental treatments.

3. Results

Prior to the experiment there were no differences in mass (χ^2 = 0.34, p =0.56) haematocrit, (χ^2 = 1.3, p =0.25) or tarsus length (χ^2 = 1.1, p =0.19) between subordinate and dominant birds, suggesting that dominant behaviours were not simply determined by size or condition.

3.1. Haematocrit, body mass and parasitaemia

To investigate differences in parasite-mediated morbidity, we first analyzed the haematocrit, body mass and parasitaemia. Table 1 shows the output from our model explaining haematocrit post-infection. Notably, there was a significant three-way interaction between dominance status, infection and parasite occurrence in competitors, though the four-way interaction with time was not-significant (time*infection*dominance*occurrence $F_{1,351.9}$= 0.60, p=0.44). Our pre-experimental prediction focussed on differences in parasite virulence between infected subordinate and dominant birds depending on the occurrence of parasites in their competitors. Therefore to avoid making a large number of post-hoc comparisons we computed least-squares means for infected birds only based on the results of our final GLMM, and performed pairwise simple comparisons tests for significant differences between these groups of interest. We found that haematocrit values differed significantly between infected subordinates competing with infected dominant birds, and infected subordinates competing with non-infected dominant birds (S+(D+) vs S+(D-): estimate = -3.8 ± 1.92, t= - 1.97, Tukey-adjusted p = 0.043). We also found a non-significant trend for a difference in haematocrit between infected dominants competing with either infected or non-infected subordinates (D+(S+) vs D+(S -): estimate = 3.3 ± 1.98, t= 1.67, Tukey adjusted p = 0.09). Figure 1 shows that contrary to our prediction, infected subordinate birds fared worse when housed with infected dominants. Moreover, we predicted occurrence-mediated

differences would be ameliorated in dominant birds. In direct contrast to subordinate birds, infected dominants had higher haematocrit when kept with infected subordinates. This result neatly indicates that the infection status of different individuals within a social group does modify parasite virulence, and that this occurrence-mediated change in virulence differs depending on social status.

Interestingly, we found that the interaction occurrence*dominance was verging on significance (without the three-way interaction $p = 0.013$), and explained changes in haematocrit better than infection*occurrence and infection*dominance. The term infection*occurrence should test differences between individuals in the outcome of infection depending on infection status of competitors as demonstrated previously (Bedhomme et al. 2005), and it is notable that this was not significant on its own here. We suggest that our three way interaction shows that in social animals the prevalence-mediated virulence proposed by Bedhomme et al. (2005) do occur, but these are likely to be extremely dependent on social rank, and may be masked when this is not considered. Furthermore, that occurrence*dominance seems important even without considering infection suggests that the cost of conspecific competition is high for these canaries, and that the infection status of competitors may partly determine these costs, even in uninfected birds.

In spite of the effect of dominance and parasite occurrence in competitors on haematocrit, we found no evidence that either factor or their interactions affected body mass (dominance $F=1.4$, $p=0.22$; occurrence $F=0.02$, $p=0.88$). There was a significant interaction between time and infection on body mass ($F_{1, 359.3} = 7.15$, $p = 0.0079$). Figures 2 shows that infected birds suffered a greater loss of mass throughout the acute phase of infection than non-infected birds.

Parasitaemia was unaffected by either dominance (d.f. = 68, F=0.72, p= 0.40) or parasite prevalence (d.f. = 68, F=0.66, p= 0.42).

3.2. Mortality

We found that infection explained much of the mortality observed in the experiment (Table 2). Independent of infection, we found a significant interaction between dominance status and parasite occurrence on mortality. This interaction was driven by differences between dominant and subordinate birds (whether infected or not) competing with uninfected competitors. In these conditions mortality was always higher in dominant than subordinate birds, regardless of infection status (simple comparisons test: t=2.8, adjusted p=0.035). This suggests dominant birds always have greater competition mediated costs when their competitors are uninfected.

3.3. Behaviour

We found a significant interaction between dominance and parasite occurrence on the change in aggression ($F_{1,78}$ =7.88, p = 0.0063). There was no effect of infection ($F_{1,78}$=0.03, p=0.87). Least squares means comparisons show that dominant birds (whether infected or uninfected) competing with infected subordinate birds decreased aggression, whereas dominants competing with uninfected subordinates did not (table 3). Subordinate birds did not significantly differ in their behaviour from one another. It is important to emphasize that changes in aggression were always small, and thus dominant birds did not become subordinate in terms of their behaviour (mean frequency of aggression in pre-experiment trials: dominants 3.79 ± 0.29, subordinates 1.54 ± 0.18).

4. Discussion

Bedhomme et al. (2005) showed that parasite virulence depends not just on a host's own infection, but also on the infection status of competing conspecifics. Our aim in this experiment was to assess whether parasite virulence in male adult canaries was dependent on the infection status of competitors in the social group, when individuals were competing for the same food resource. Moreover, given that canary social groups are characterised by the establishment of dominance relationships among the members, we added social status to this theoretical framework and predicted that a bird's dominance and infection status would interact with the social and infection status of competitors (parasite occurrence) to differentially affect virulence. While infection, dominance status, and the parasite occurrence did significantly interact to produce different mortality and morbidity for these canaries, the patterns were not always as predicted.

We found that social status, parasite occurrence, and infection status interacted to significantly affect haematocrit. Our prediction was that infected subordinate birds competing with uninfected dominant birds would suffer greater morbidity than infected subordinate birds competing with infected dominant birds, but this parasite occurrence-mediated difference would be ameliorated in dominant birds. In fact, we found that infected subordinate birds competing with non-infected dominants had significantly higher haematocrit than those competing with infected dominants, in direct contrast with our prediction. Furthermore, rather than the difference being ameliorated in dominant birds, infected dominants competing with uninfected subordinates had lower haematocrit (i.e. were more anaemic), than those competing with infected subordinates; the opposite of our results for subordinate birds. It is unclear exactly why this should be; certainly we did not find that infected dominant birds became more aggressive towards subordinate birds. On the contrary,

372 infected dominant birds competing with infected subordinates actually showed a small
373 decrease in aggression. Although we set out specifically to test whether social rank, parasite
374 occurrence in competitors and infection had an interactive effect on morbidity in this study, it
375 is interesting to note that without considering social rank we found no evidence of of the
376 prevalence dependant impacts of parasitism demonstrated by Bedhomme et al. (2004). This
377 shows that for some animals, social context is of considerable importance when predicting the
378 outcome of infection. That the effects of the same parasite may differ markedly between
379 subordinate and dominant birds, depending on its occurrence within a social group, is a
380 fascinating development for our understanding of both parasite virulence, and the costs and
381 benefits of group living.

382 It is interesting to speculate as to why the dominant and subordinate birds differed in
383 their responses to the infection in a manner we did not predict. Our pre-experimental
384 predictions were strictly based on assumptions about differences in competitive ability
385 between dominant and subordinate birds, and the effects these would have on prevalence-
386 dependent costs of parasite virulence (Bedhomme et al. 2005). Our results suggest a more
387 complicated scenario than this. One consideration is that in our experiment the birds
388 competed for an identical food resource within each cage. Following infection, it is likely that
389 energetic demands changed, in particular an increase in food requirements to cope with the
390 energetic costs of infection. The costs of this increase in requirement to compete for food will
391 depend upon the motivation of conspecific competitors to feed simultaneously. If infection
392 increased the food demands of infected dominants, this may explain why the infected
393 subordinates housed with them paid higher costs. We predicted that infected subordinates
394 would pay greater competition-mediated costs than dominants. We did not find any evidence
395 to support this, in terms of either reduction in body mass or haematocrit. One consideration is
396 that we classified birds as dominant or subordinate in order to achieve our balanced

397 experimental design. Although we feel these classifications were justified, it is very likely that
398 there is little difference between the third ranked dominant and fourth ranked subordinate
399 bird, at least in comparison to top-ranked dominant and last-ranked subordinate birds. It was
400 not within the scope of this experiment to address these differences, but in future, examining
401 the effects of infection across a gradient of behavioural profiles may further our knowledge on
402 the effects of competition and social behaviour on parasite virulence.

403 Unlike haematocrit, we found no evidence that parasitaemia was modified by
404 infection, dominance, or parasite prevalence in the social group. One potential explanation for
405 this discrepancy is that while plasmodium infection does induce a haematocrit reduction in
406 canaries (Cellier-Holzem et al. 2011; Spencer et al. 2005; Bichet et al. 2012; Cornet et al. in
407 review), haematocrit will also be subject to modification by other factors (Fair et al. 2007). In
408 our case, the limited quantity of seed provided could result in changes in red blood cell
409 production, in addition to the direct destruction of red blood cells caused by the parasite. Our
410 results for haematocrit might reflect the overall costs of the infection, diet and competition
411 between birds, whereas parasitaemia may reflect a more specific difference in the
412 physiological responses to infection. Of course, this does not detract from our results for
413 haematocrit, since in a natural setting parasite virulence will always be determined by the
414 overall environment of a host; this will include diet (e.g. Tseng et al. 2006, Cornet et al. in
415 review), social status, parasite prevalence (e.g. Bedhomme et al. 2005), and temperature (e.g.
416 Murdock et al. 2012) among other factors.

417 There are several other physiological differences between dominant and subordinate
418 birds that may affect parasite virulence in addition to/instead of the ability to access or
419 monopolise food resources. For example, dominant and subordinate birds are known to have
420 differences in circulating levels of androgens, or glucocorticoid stress hormones, often with

higher levels of hormones with apparently harmful secondary effects in dominant birds than subordinates (e.g. Goymann and Wingfield 2004). Immune responsiveness may also depend on social status, with some studies reporting dominant individuals as having a better immune response than subordinates (e.g. in goats, Ungerfeld and Correa 2007), and others reporting subordinates as having a higher investment in immune responsiveness than dominants (e.g. in voles, Li et al. 2007). In house finches (*Carpodacus mexicanus*), Hawley et al. (2007), demonstrated that dominants and subordinates differ in their responsiveness to various immune challenges, though dominant males were better able to resist an experimental infection with *Mycoplasma* than subordinates. We cannot rule out a role for these physiological differences in explaining some of the differences observed between dominant and subordinate birds. However, it has been shown that behavioural processes such as aggression may mediate some of these physiological changes (Hawley 2006), so the extent to which differences between dominant and subordinate birds are determined by pre-existing, unavoidable differences in physiology or by physiological changes brought about by behavioural differences is a question to be addressed further.

It is clear from our results that for organisms with complex social hierarchies the interactions among infection, social status and the prevalence of infection in the flock are likely to be multifaceted. We found evidence that change in haematocrit was modified by a bird's social status, and the infection status of competitors. This represents the first confirmation of an interaction between social rank and parasite-prevalence dependent virulence in birds. This has important implications for our future understanding of the evolutionary processes that shape both parasite virulence, and group living.

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Figure Legends

Figure 1: Least Means Squares values for haematocrit in infected birds only (± S.E). The error bars are divided by the host's dominance status, then by parasite occurrence in competitors in brackets e.g. the first error bar represents infected dominant birds, kept with infected subordinates (D+(S+)).

Figure 2: Change in body mass for all birds at each measured day post experimental treatment. Error bars represent either infected or non-infected birds.

Figure 3. Least Means Squares changes in aggression (± S.E) for dominant and subordinate birds, divided by prevalence of parasite in competitors (+ infected, - non infected). Change is provided as the difference in aggressive behaviour (measured in frequency of aggressive behaviour per trial) between pre and post-experimental behavioural trials.